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[Title of Invention] Molecular Detection Method, Molecular Localization Detection Method, and Molecular Counting Method

[Number of Claim(s)] 7

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[Number of Payment Ledger] 001982

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[List of Attached Document(s)]

[Title of Article] Specification 1

[Title of Article] Drawings 1

[Title of Article] Abstract 1

[Number of General Power of Attorney] 0302311

[Title of Document] Specification

[Title of the Invention] Molecular Detection Method, Molecular Localization Detection Method, and Molecular Counting Method

[Claims]

[Claim 1] A molecular detection method comprising visualizing and identifying a chain molecule immobilized on a substrate by probing with a scanning probe microscope in solution.

[Claim 2] The molecular detection method according to Claim 1, wherein the chain molecule immobilized on the substrate is an uprightly disposed single strand molecule.

[Claim 3] The molecular detection method according to Claim 2, wherein the uprightly disposed single strand molecule is a nucleic acid, a peptide nucleic acid, a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer, or an analog thereof.

[Claim 4] The molecular detection method according to Claim 1, wherein the chain molecule immobilized on the substrate is a multiple strand molecule comprising an uprightly disposed single strand molecule and at least one chain molecule that can bind to the single strand molecule.

[Claim 5] The molecular detection method according to Claim 4, wherein the multiple strand molecule is a complex of one or more types of molecules selected from a nucleic acid, a peptide nucleic acid, a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer, or an analog thereof.

[Claim 6] A molecular localization detection method comprising detecting a molecule by the method according to any one of Claims 1 to 5, and counting the number of detected chain molecules per unit area, thus giving molecular localization information.

[Claim 7] A molecular counting method comprising detecting a molecule by the method according to any one of Claims 1 to 5, and counting the number of detected chain molecules per unit area.

[Detailed Description of the Invention]

[0001]

[Technical Field to which the Invention Pertains]

The present invention relates to a molecular detection method, localization detection method, and counting method.

More particularly, it relates to a method that enables a chain molecule standing upright to be visualized and identified by probing a molecule by means of a probe of a scanning probe microscope.

[0002]

[Background Art]

When molecules are immobilized on a substrate, there is no method confirming at what density the molecules are immobilized or in what conditions the molecules are immobilized. For example, measuring absorbance or fluorescence intensity by staining a protein molecule immobilized on a substrate, such as a microtiter plate or a protein chip, which is used for enzyme-linked immunosorbent assay (ELISA), with a dye, etc. having an affinity for the protein molecule, only an average value for the protein concentration per unit area can be obtained, and molecular localization information cannot be obtained.

Furthermore, a DNA chip is produced by a method in which each base is added on a substrate thereby synthesizing individual DNAs and a DNA microarray is produced by a method in which single stranded DNAs are spotted on a substrate. However, there is no technique for examining, at the molecular level, whether or not the single strand DNAs are uniformly immobilized on a specified area on the substrate.

[0003]

With regard to means for obtaining information about localization of molecules, there is observation using an electron microscope, etc. However, since this observation is carried out under vacuum, in the case of biopolymers, the structure thereof will be destroyed and observation is not possible, or they stick to the substrate, thus making it impossible to distinguish them from the substrate.

[0004]

In addition, a technique of imaging a material bonded on the surface of a substrate by a spectroscopic method has been employed in recent years, but since due to the structure of the equipment, the field of vision of the measurement is wide, it is

difficult to obtain localization information at the molecular level.

[0005]

In order to visualize a molecule and obtain information about its shape, observation using a scanning probe microscope is most preferable. With regard to a nucleic acid, the helical structure of a double strand nucleic acid has been confirmed by observation using a scanning probe microscope (ref. e.g. Non-patent References 1 and 2). It also becomes possible to distinguish between a single strand DNA and a double strand DNA by atomic force measurement using an atomic force microscope (ref. e.g. Non-patent References 3). However, in these techniques, since the nucleic acid is made to adsorb parallel to the substrate, the degree of freedom of the molecule is restricted, and it must be said that the molecule is in an inactive state, which is not desirable for a subsequent reaction.

[0006]

In inspection by the scanning probe microscope, there is an obstacle in the horizontal direction during probing to distinguish between a single strand DNA and a double strand DNA. With regard to conditions for high-resolution observation by the scanning probe microscope, a material needs to be constantly scanned by the one point of a probe tip. In such conditions, the side of the probe contacts with the material, which results in deterioration of resolution.

[0007]

In the above-mentioned DNA chip, ELISA, etc., molecules are immobilized on a substrate while maintaining a preferable state, such as an active state. That is, by making the molecules stand upright on the substrate, the degree of freedom of the molecules is increased, and the reaction site is made more open, thus improving the reactivity. However, unless the molecules are uniformly immobilized on an intended section on the substrate, quantitative analytical performance cannot be exhibited.

[0008]

As a method for confirming whether or not molecules are uniformly immobilized, observation using a scanning probe microscope is known. However, an uprightly disposed single strand molecule is easy to bend because of its softness.

When the uprightly disposed molecule bends, the height of the molecule is detected lower than the actual height of the molecule. Furthermore, the bent molecule overlapped each other affects on the resolution in lengthwise and crosswise direction, which results in information about localization of the molecules may not be obtained.

[0009]

On the other hand, from the viewpoint of a measurement environment, since there is a difference in environments between measurement in air and in solution, molecules stick to the substrate in air, but move around freely in solution. When molecules are measured in air, adjacent molecules are intertwined with each other and stick to the substrate, and individual molecules cannot be identified.

[0010]

[Non-patent Reference 1]

T. P. Beebe, Jr., T. E. Wilson, D. F. Ogletree, J. E. Katz, R. Balhorn, M. B. Salmeron and W. J. Siekhaus, Science 243, 370(1989)

[Non-patent Reference 2]

R. J. Driscoll, M. G. Youngquist and J. D. Baldeschwieler, Nature 346, 294 (1990)

[Non-patent Reference 3]

J. Wang and A. J. Bard, Anal. Chem. 73, 2207 (2001)

[0011]

[Problems to be Solved by the Invention]

The present invention relates to provide a molecular detection method, localization detection method, and counting method that enable chain molecules immobilized on a substrate to be clearly identified.

The present invention further relates to provide an accurate localization detection method for molecules on a substrate, the methods involving making an upright single strand molecule rigid so that it can withstand a scanning probe microscope, and carrying out measurement in solution so that the upright molecule does not stick to the substrate.

[0012]

[Means for Solving the Problem]

The above-mentioned problems are solved by the following present invention.

(1) A molecular detection method comprising visualizing and identifying a chain molecule immobilized on a substrate by probing with a scanning probe microscope in solution.

[0013]

(2) The molecular detection method according to (1), wherein the chain molecule immobilized on the substrate is an uprightly disposed single strand molecule.

[0014]

(3) The molecular detection method according to (2), wherein the uprightly disposed single strand molecule is a nucleic acid, a peptide nucleic acid, a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer, or an analog thereof.

[0015]

(4) The molecular detection method according to (1), wherein the chain molecule immobilized on the substrate is a multiple strand molecule comprising an uprightly disposed single strand molecule and at least one chain molecule that can bind to the single strand molecule.

[0016]

(5) The molecular detection method according to (4), wherein the multiple strand molecule is a complex of one or more types of molecules selected from a nucleic acid, a peptide nucleic acid, a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer, or an analog thereof.

[0017]

(6) A molecular localization detection method comprising detecting a molecule by the method according to any one of (1) to (5), and counting the number of detected chain molecules per unit area, thus giving molecular localization information.

[0018]

(7) A molecular counting method comprising detecting a molecule by the method according to any one of (1) to (5), and counting the number of detected chain molecules per unit area.

[0019]

[Modes for Carrying Out the Invention]

The present invention is explained in detail below.

In the present invention, a chain molecule, which is a detection target, generally has a length that is greater than the roughness of a substrate, and is usually either a single strand molecule or a multiple strand molecule. When the detection target is the single strand molecule, since the multiple strand molecule is rigid, it is preferable to form a complex (a multiple strand molecule) containing the uprightly disposed target single strand molecule and one or more complementary molecules thereto so as to distinguish the complex from adjacent molecules and clearly detect the molecule.

[0020]

<Arrangement of single strand molecule>

In the present invention, the single strand molecule is generally a nucleic acid such as a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), an artificial nucleic acid having adenine, thymine, cytosine, guanine, uracil, or hypoxanthine, or a nucleic acid derivative, or a peptide nucleic acid (PNA). Furthermore, one having a complementary molecule such as a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer (e.g. polymethacrylic acid, polyacrylic acid, polyvinylimidazole, polystyrenesulfonic acid, polyallylamine, polythiopheneacetic acid, or polypyridylacetylene), or an analog thereof is preferable since the above-mentioned multiple strand molecule may be formed, but the single strand molecule is not limited thereto. It is also possible to use a synthetic polymer such as polyphenol, polyester, polyethylene glycol, polyamide acid, polyvinylpyrrolidone, polyacrylamide, or polyvinylalcohol.

With regard to the chain molecule, for example, one having in part a branched structure or a network structure may be used, as long as it can be detected by the present invention.

[0021]

The above-mentioned target single strand molecule is normally disposed on

(immobilized on) a substrate in an upright state. Disposing on (immobilizing on) the substrate referred to in the present invention means immobilizing by means of indirect bonding via a linker, or any physical adsorption or chemical bonding including electrostatic binding, hydrophobic binding, hydrophilic binding, ionic bonding, and hydrogen bonding.

[0022]

The substrate referred to above generally includes a plate, a bead, a well, a membrane, and a film, which are made of a material such as plastic, glass, or metal, and is usually a plate.

[0023]

<Formation of multiple strand molecule>

In the method mentioned above, a single strand molecule is uprightly disposed relative to the substrate. However, since the single strand molecule bends on its own, if there are adjacent molecules they overlap each other, and it might become difficult to recognize them as individual molecules. It is therefore preferable to add one or more types of single strand molecules that are complementary to the arranged single strand molecule, thus forming a multiple strand molecule. For example, in the case a single strand molecule is a DNA, by adding a single strand DNA having a complementary base sequence, a double strand DNA is formed. By so doing, the molecular structure becomes rigid, resistance to contact with a probe can be introduced, and detection sensitivity improves, which are preferable.

[0024]

In the present invention, the multiple strand molecule is generally a complex containing the above-mentioned target molecule and a one or more chain molecule which can be bond to the target molecule, for example, a complex formed by interaction between molecules such as the target molecule and the complementary molecule. Accordingly, the multiple strand molecule referred to in the present invention is preferably a complex of one or more types of molecules selected from a nucleic acid, a peptide nucleic acid, a peptide, a glycopeptide, a protein, a glycoprotein, a polysaccharide, a synthetic polymer, and an analog thereof. Examples

of the multiple strand molecule referred to in the present invention include a double strand DNA, a double strand RNA, a double strand PNA, a hybrid of DNA and RNA, a hybrid of DNA and PNA, a hybrid of RNA and PNA, a hybrid of an artificial nucleic acid having adenine, thymine, cytosine, guanine, uracil, or hypoxanthine or a nucleic acid derivative with DNA, RNA, or PNA, and a complex such as a triple strand nucleic acid.

[0025]

The conditions under which the multiple strand molecule is formed are not particularly limited, and it may be carried out in accordance with a standard method. For example, formation of a double strand DNA, that is, hybridization, is carried out in an aqueous solvent containing a salt such as sodium chloride, potassium chloride, ammonium chloride, sodium acetate, potassium acetate, ammonium acetate, or a buffer solution.

[0026]

<Scanning probe microscope>

A scanning probe microscope used in the present invention is a general term for scanning probe microscopes that enable observation at the atomic level, and includes a scanning tunneling microscope (STM) and an atomic force microscope (AFM). AFM can be roughly divided into repulsive mode, attractive mode, tapping mode (' tapping mode ' is a registered trademark of Digital Instruments, Inc., Santa Barbara, California, USA), etc. Research in this field is still in progress, and new microscopes are being developed. The scanning probe microscope referred to in the present invention is not limited to those currently known and includes microscopes that will be developed in the future as long as they have a resolution at the atomic level.

The probing method itself may be a standard method that is used with the above-mentioned microscopes, and it is preferable to carry out AFM observation in solution.

[0027]

<Solution>

There is a difference between the environment in air and that in solution; in air, adjacent molecules are intertwined with each other and stick to a substrate, but in

solution molecules move around freely.

In the present invention, by carrying out observation by means of a scanning probe microscope in solution, an image of the upright molecule on the substrate can be obtained. Since a single strand molecule bends, and a double strand molecule is rigid and not to bend, the two can be distinguished by height. The double strand molecule is resistant to the influence of an obstacle in the horizontal direction.

[0028]

The solution used in the detection method of the present invention is not particularly limited as long as the detection target can exist stably, and it is preferable to use an aqueous solution containing a salt such as sodium chloride, potassium chloride, ammonium chloride, sodium acetate, potassium acetate, or ammonium acetate, or a buffer solution, which are used when forming the above-mentioned multiple strand molecule.

[0029]

By estimating the number of chain molecules having a height that is equal to or greater than a certain threshold value in the above-mentioned method, information about the localization of molecules can be obtained. By counting the number of chain molecules probed per unit area, the number of molecules can be counted.

[0030]

Example 1

Examples of the present invention are explained below.

[0031]

<Substrate used>

As a substrate, a plastic substrate (1.5 cm × 1.5 cm) into which carboxyl groups had been introduced was used.

[0032]

<Immobilization of dT_{20}>

Firstly, a 25 mM EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl) solution was prepared using a 0.1 M MES (2-(*N*-morpholino)ethanesulfonic acid) buffer solution whose pH had been adjusted to 6.0 using sodium hydroxide. Subsequently,

by use of this solution and dT_{20} (5 ' -NH_2-$(CH_2)_6$-(thymidine 5 ' -monophosphate)$_{20}$), a solution (1.3 μM dT_{20}/EDC solution) was prepared; 100 to 150 μL of this solution was dropped on the substrate, and after a reaction by incubating at 60°C for 6 hours, the substrate was washed with pure water so as to remove excess solution.

[0033]

<Observation of single strand nucleic acid>

The substrate with the single strand DNA immobilized thereon was immersed in a TE buffer solution (Tris-EDTA buffer solution, 10 mM Tris, 1 mM EDTA, pH 7.8, containing 0.5 M sodium chloride), and undulations of the surface of the substrate with dT_{20} immobilized thereon were imaged by means of an atomic force microscope. As the atomic force microscope, a model SPI3800N, manufactured by Seiko Instruments, Inc., was used, and observation was carried out in DFM mode by scanning a region of 500 nm x 500 nm.

[0034]

FIG. 3 is an image of the substrate observed with the atomic force microscope. Undulations were hardly observed.

[0035]

FIG. 4 is an image of the substrate with dT_{20} immobilized thereon prepared by the method above observed with the atomic force microscope. Particles having a height of about 5 nm overlapped each other and they were unclearly observed.

[0036]

<Formation of double helix>

As a complementary single strand nucleic acid, dA_{20}-FAM (5 ' -5-carboxy-fluorescein-$(CH_2)_6$-(2 ' -deoxyadenosine 5 ' -monophosphate)$_{20}$) was used. This dA_{20}-FAM was dissolved in a TE buffer solution (20 pmol/μL). 100 to 150 μL of this solution was dropped on the substrate with dT_{20} immobilized thereon. One hour later, excess dA_{20}-FAM solution was removed by washing with a TE buffer solution, the substrate was immersed in a TE buffer solution, and undulations of the surface of the substrate with dT_{20} immobilized thereon were imaged by means of the atomic force microscope.

[0037]

FIG. 5 is an image of the double strand nucleic acid formed by the above-mentioned method observed with the atomic force microscope. Particles having a height of 7 to 8 nm were clearly and regularly observed.

[0038]

<Image of double helix formed in air>

The surface of a substrate on which the immobilized dT_{20} and dA_{20}-FAM formed a double helix was imaged in air by means of the atomic force microscope.

[0039]

FIG. 6 is an image of the double strand nucleic acid formed by the above-mentioned method observed with the atomic force microscope in air instead of being immersed in a TE buffer solution. It can be confirmed that, unlike in FIG. 4, the height could not be discriminated.

[0040]

FIG. 7 is a schematic diagram showing line profiles in FIG. 4 and FIG. 5. (a) shows a single strand DNA immobilized on the substrate, and (b) shows a double strand DNA having identical numbers of bases immobilized on the substrate.

[0041]

Example 2

Particle analysis was carried out for the substrate used, the substrate with dT_{20} immobilized thereon formed by the above-mentioned method, and the substrate on which a double helix of immobilized dT_{20} and dA_{20}-FAM was formed by the above-mentioned method.

[0042]

The particle analysis was carried out using software included with the model SPI3800N, manufactured by Seiko Instruments, Inc. The number was determined by setting a threshold value of 7.5 nm and excluding particles having a particle area of equal to or less than 50 nm^2.

[0043]

FIG. 8 is a particle analysis image of the substrate. Dark spots denote counted particles, and pale spots denote excluded particles. The number of particles

having a height of equal to or greater than 7.5 nm was 14 in a region of 500 nm x 500 nm.

[0044]

FIG. 9 is a particle analysis image of the substrate with dT_{20} immobilized thereon. Dark spots denote counted particles, and pale spots denote excluded particles. The number of particles having a height of equal to or greater than 7.5 nm was 17 in a region of 500 nm x 500 nm.

[0045]

FIG. 10 is a particle analysis image of the substrate on which the double helix of immobilized dT_{20} and dA_{20}-FAM was formed. Dark spots denote counted particles, and pale spots denote excluded particles. The number of particles having a height of equal to or greater than 7.5 nm was 250 in a region of 500 nm x 500 nm. It can be confirmed that the number of molecules can thus be obtained by counting.

[0046]

[Effects of the Invention]

As described above, in accordance with the method of the present invention, chain molecules immobilized on a substrate can be clearly detected, which has conventionally been difficult. In particular, outstanding effects in which, by probing uprightly disposed single strand molecules by means of a scanning probe microscope, the molecules can be identified and information about localization can be obtained; or in which, by binding a complementary single strand molecule to this single strand molecule so as to form a rigid multiple strand molecule, a clearer molecular image can be obtained and accurate information about localization can be obtained, etc, can be recognized.

[Brief Description of the Drawings]

[Drawing 1]

FIG. 1 is a space filling model of a single strand DNA immobilized on a substrate.

[Drawing 2]

FIG. 2 is a space filling model of a double helix DNA formed from a single strand DNA immobilized on a substrate and a DNA with bases that form complementary pairs therewith.

[Drawing 3]

FIG. 3 (a) is an image of a plastic substrate observed with an atomic force microscope and FIG 3 (b) is a line profile at a given point.

[Drawing 4]

FIG. 4 (a) is an image of a plastic substrate having dT_{20} immobilized thereon observed with an atomic force microscope, and FIG. 4 (b) is a line profile at a given point.

[Drawing 5]

FIG. 5 (a) is an image of the sample shown in FIG. 4 to which dA_{20}-FAM has been added so as to form a double helix observed with an atomic force microscope, and FIG. 5 (b) is a line profile at a given point.

[Drawing 6]

FIG. 6 is an image of the sample shown in FIG. 5 observed in air with an atomic force microscope.

[Drawing 7]

FIG. 7 (a) is a schematic diagram of a single strand DNA immobilized on a substrate when observed with an atomic force microscope, and FIG. 7 (b) is a schematic diagram of a double strand DNA having the same number of bases when observed with an atomic force microscope.

[Drawing 8]

FIG. 8 (a) is a particle analysis diagram of the substrate shown in FIG. 3, and FIG. 8 (b) is a histogram in which the abscissa is the proportion of particles (number of

particles) and the ordinate is the particle height.

[Drawing 9]

FIG. 9 (a) is a particle analysis diagram of the substrate with dT_{20} immobilized thereon shown in FIG. 4, and FIG. 9 (b) is a histogram in which the abscissa is the proportion of particles (number of particles) and the ordinate is the particle height.

[Drawing 10]

FIG. 10 (a) is a particle analysis diagram of the substrate shown in FIG. 5, on which the double helix has been formed, and FIG. 10 (b) is a histogram in which the abscissa is the proportion of particles (number of particles) and the ordinate is the particle height.

[Description of Notations]

1 Substrate

2 dT_{20} single strand nucleic acid

3 Double strand dT_{20} and dA_{20}-FAM nucleic acids

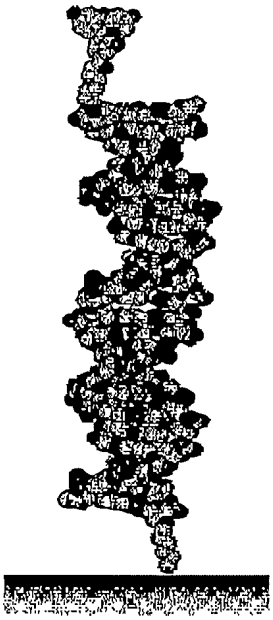
4 Shape observed for single strand DNA

5 Shape observed for double strand DNA

[Title of Document] Drawings

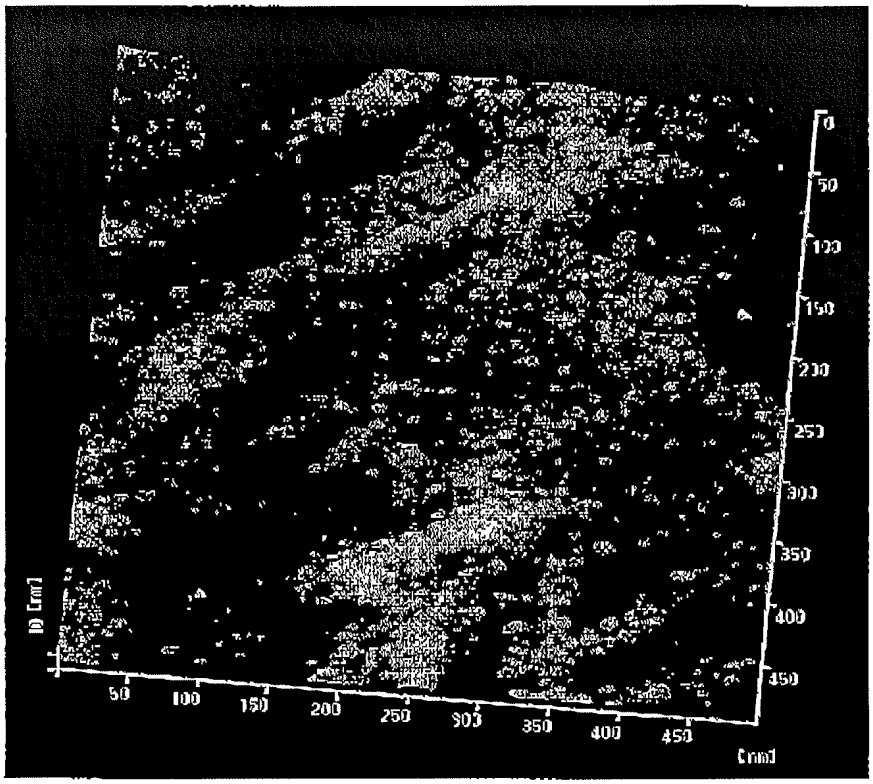
[Drawing 1]

[Drawing 2]



[Drawing 3]

(a)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100
150
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[nm]
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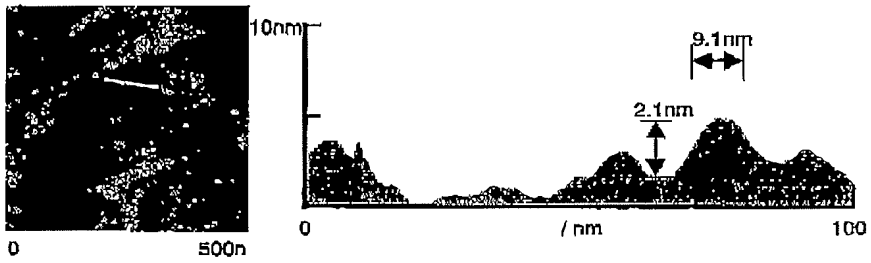
450
[nm]

(b)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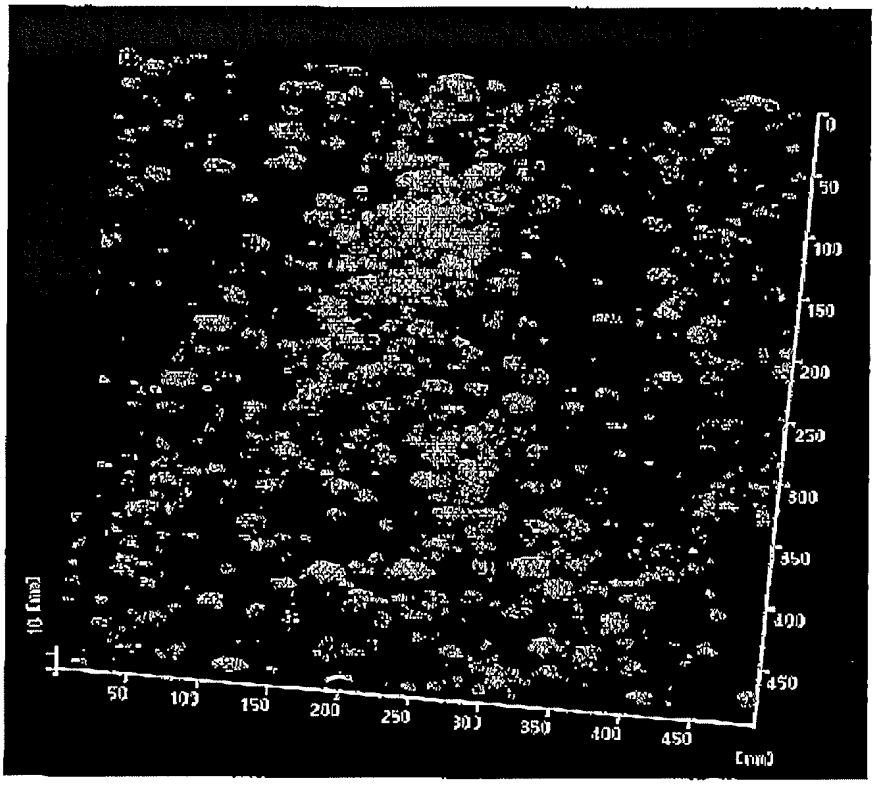
500n
0
/ nm
100

[Drawing 4]

(a)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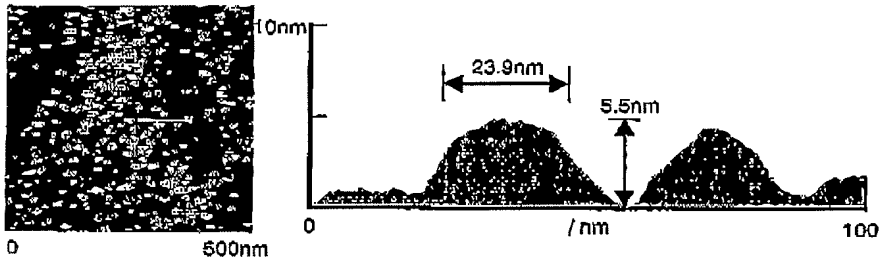
(b)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/nm
100

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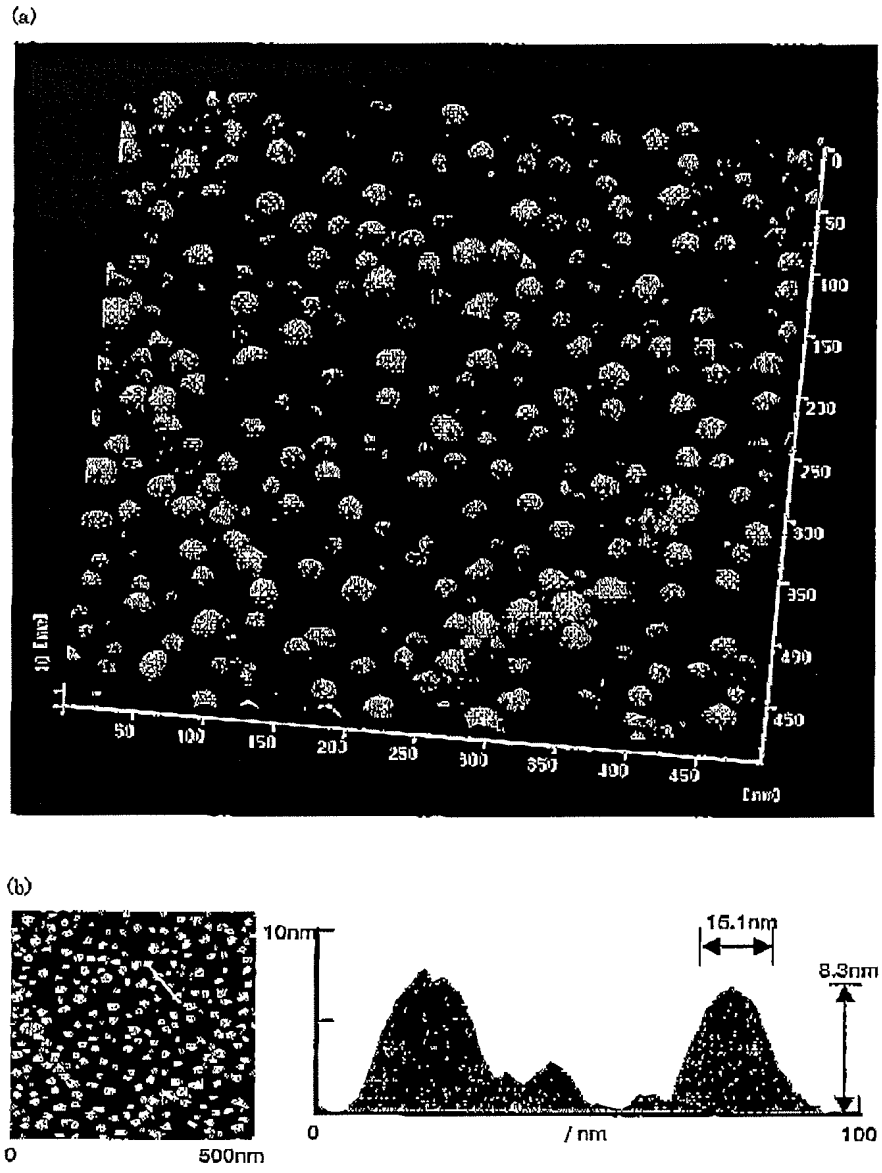
(a)
0
50
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200
250
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(b)
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0
500nm
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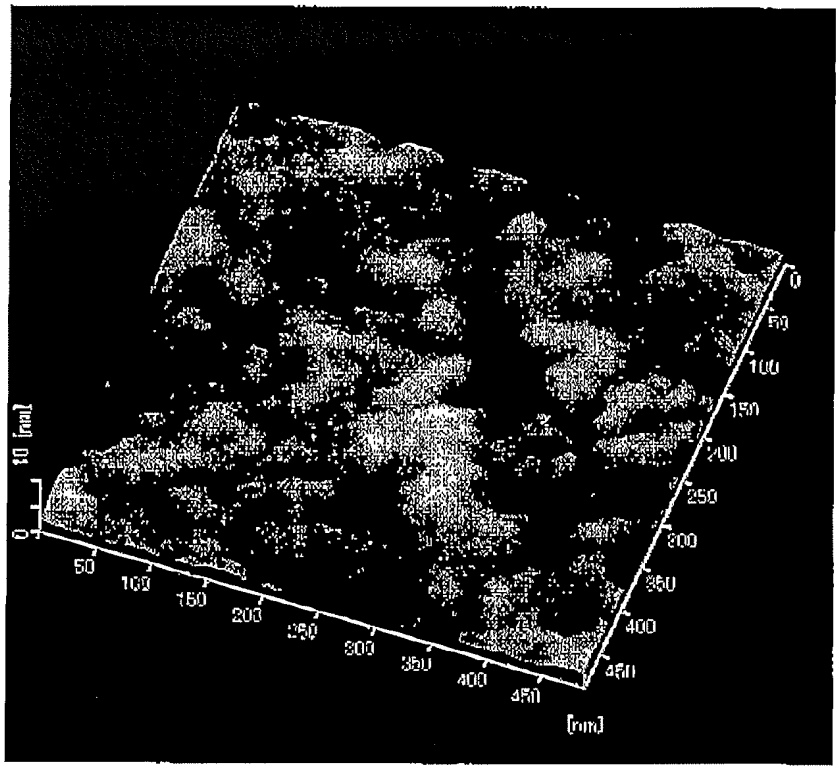
8.3nm
0
/ nm
100

[Drawing 6]



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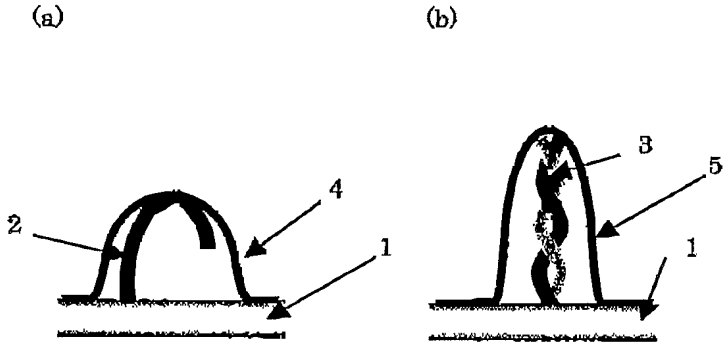

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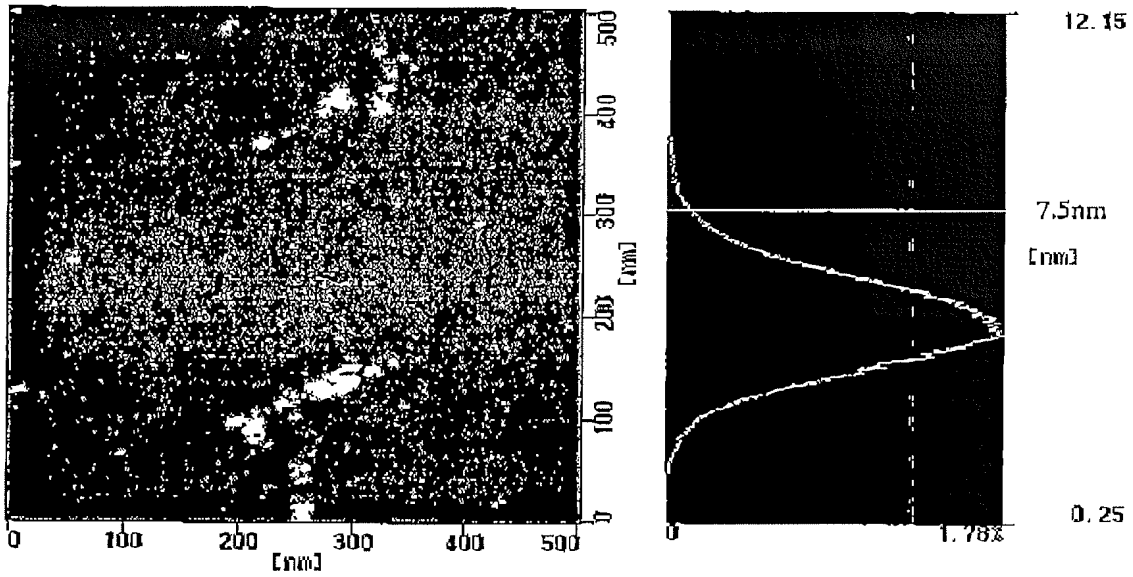

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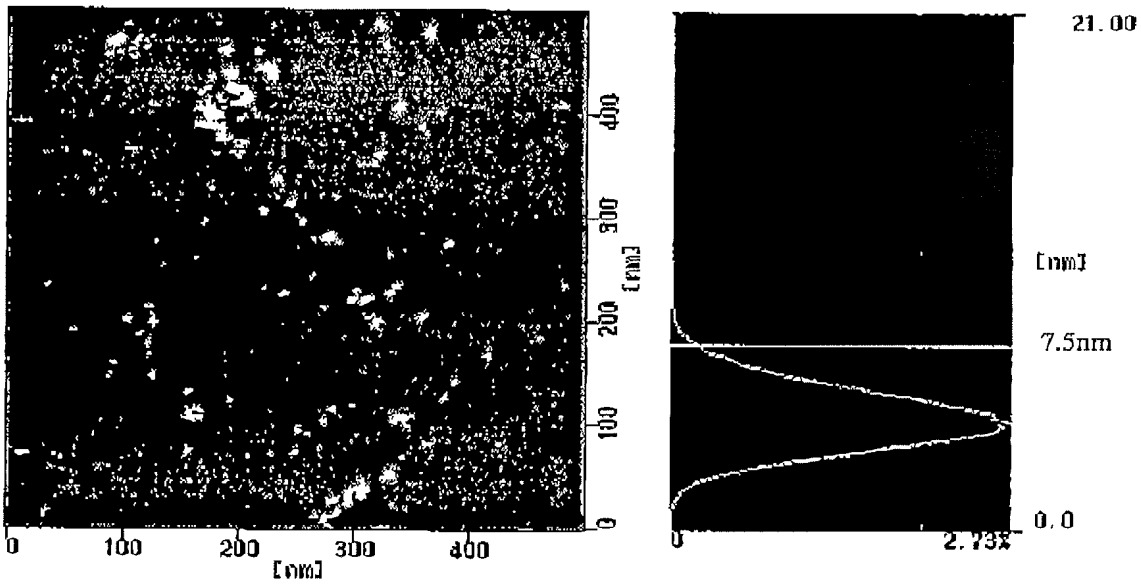
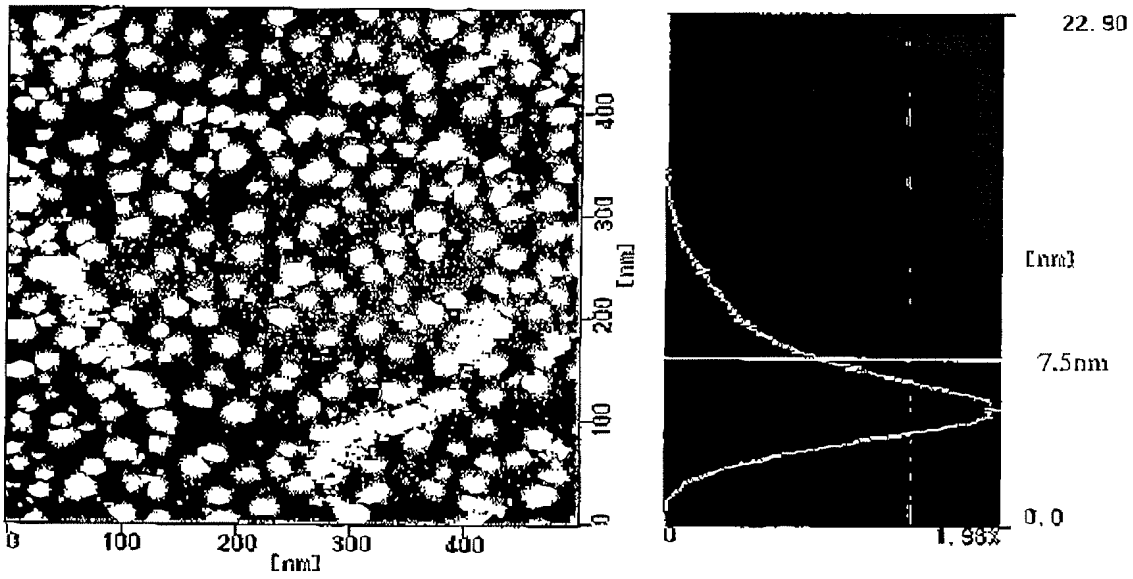


[Drawing 10]



[Title of Document] Abstract

[Abstract]

[Objects of the Invention] The present invention relates to provide a molecular detection method, localization detection method, and counting method that enable chain molecules immobilized on a substrate to be clearly identified.

[Means for Solving the Problem] A molecular detection method comprising visualizing and identifying a chain molecule immobilized on a substrate by probing with a scanning probe microscope in solution; a molecular localization detection method comprising detecting a molecule by the above-mentioned method, and counting the number of detected chain molecules per unit area, thus giving molecular localization information; and a molecular counting method comprising detecting a molecule by the above-mentioned method, and counting the number of detected chain molecules per unit area.

[Elected Drawing] Fig. 5

DECLARATION

I, Fumiko WATANABE, a citizen of Japan, c/o Miyoshi & Miyoshi of Toranomon Kotohira Tower, 2-8, Toranomon 1-chome, Minato-ku, Tokyo 105-0001, Japan, do hereby solemnly and sincerely declare:

That I am well acquainted with the Japanese language and English language; and

That the attached is a true and faithful translation made by me of the Japanese document, namely Japanese Application No. 2003-114836 to the best of my knowledge and belief.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-captioned application or any patent issuing therefrom.

This 07th day of April, 2010

Fumiko Watanabe

Fumiko WATANABE